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Nitrification and the ammonia-oxidizing communities in the central Baltic Sea water column

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4 **Nitrification and the ammonia-oxidizing communities in the central**
5 **Baltic Sea water column**

6 **Running head:** Nitrification in the central Baltic Sea

7

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21 **Key words:** Ammonia-oxidizing bacteria, ammonia-oxidizing archaea, microarray, nitrification, Baltic Sea

22 **Abstract**

23 The redoxclines that form between the oxic and anoxic water layers in the central Baltic Sea are sites of
24 intensive nitrogen cycling. To gain better understanding of nitrification, we measured the biogeochemical
25 properties along with potential nitrification rates and analyzed the assemblages of ammonia-oxidizing
26 bacteria and archaea using functional gene microarrays. To estimate nitrification in the entire water column,
27 we constructed a regression model for the nitrification rates and applied it to the conditions prevailing in the
28 area in 2008-2012. The highest ammonia oxidation rates were found in a thin layer at the top of the
29 redoxcline and the rates quickly decreased below detection limit when oxygen was exhausted. This is
30 probably because extensive suboxic layers, which are known to harbor pelagic nitrification, are formed only
31 for short periods after inflows in the Baltic Sea. The nitrification rates were some of the highest measured in
32 the water columns, but the thickness of the layer where conditions were favorable for nitrification, was very
33 small and it remained fairly stable between years. However, the depth of the nitrification layer varied
34 substantially between years, particularly in the eastern Gotland Basin (EGB) due to turbulence in the water
35 column. The ammonia oxidizer communities clustered differently between the eastern and western Gotland
36 Basin (WGB) and the composition of ammonia-oxidizing assemblages correlated with the environmental
37 variables. The ammonia oxidizer community composition was more even in the EGB, which may be related
38 to physical instability of the redoxcline that does not allow predominance of a single archetype, whereas in
39 the WGB, where the position of the redoxcline is more constant, the ammonia-oxidizing community was less
40 even. Overall the ammonia-oxidizing communities in the Baltic Sea redoxclines were very evenly distributed
41 compared to other marine environments where microarrays have been applied previously.

42

43

1. Introduction

The Baltic Sea is one of the largest brackish water basins (415 200 km²) in the world and subject to severe eutrophication (HELCOM 2009). The high nutrient load from the drainage basin and salinity stratification caused by positive freshwater balance have led to formation of widespread anoxic areas in the deep basins, which are separated by sills that prevent an even flow of water to the bottom areas. The widest anoxic basin in the central Baltic Sea is the Gotland Deep and the deepest the Landsort Deep (Figure 1). These basins are characterized by suboxic transition zones, redoxclines, which form in the area between the oxygenated surface and the euxinic bottom water. Unlike in many other oxygen deficient zones (ODZ), the redoxcline intermittently disappears in the central Baltic Sea due to inflow of saline (≥ 17) and oxygen rich water from the North Sea through the Danish Straits. During such events, termed as Major Baltic Inflows (MBI), the sulfidic water in the bottom of the deepest basins is replenished with oxygen (O₂) and the redoxcline disappears. MBIs occur mainly during winter and since the mid-1970s the frequency of MBIs has decreased to almost decadal, which has led to a long-term stagnation and made anoxia a nearly permanent feature of the central Baltic Sea (Schinke and Matthäus, 1998). In addition to MBIs, there is also smaller scale mixing in the water column which occurs during stagnation. The drivers for the small scale mixing are not well understood, but they are in general a result of complex hydrodynamic processes such as upwelling, boundary mixing, Kelvin-Helmholtz and other shear instabilities and internal wave breakings (Zhurbas and Paka, 1999, Kuzmina *et al.*, 2005, Reissmann *et al.*, 2009, van der Lee and Umlauf 2011).

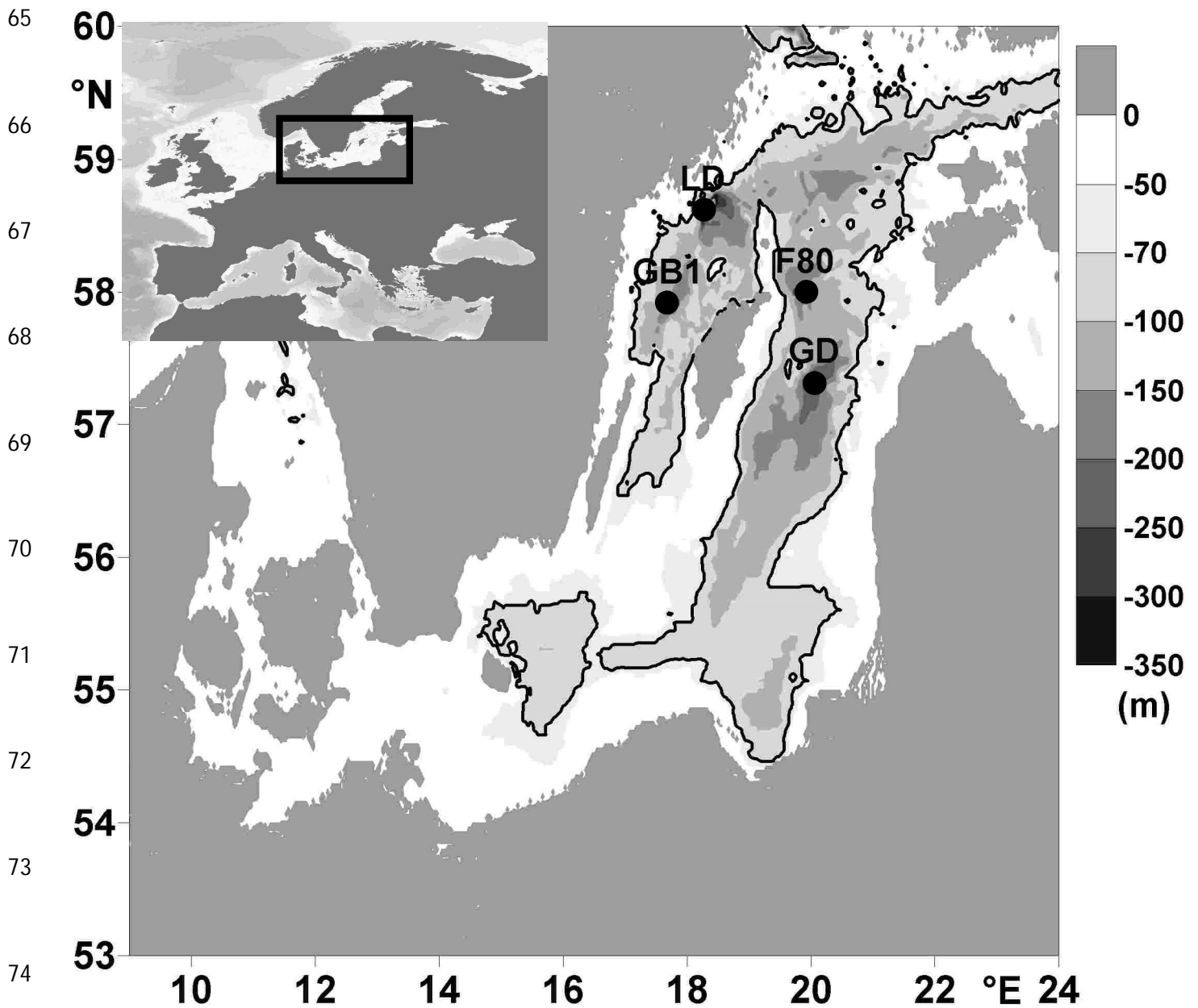


Figure 1. Topography of the Baltic Proper and the position of the sampling stations (LD, GB1, GD, and F80). GD is located in the Eastern Gotland Basin, LD and GB1 in the Western Gotland Basin and F80 in the Farö deep. The full line marks the 70 m depth contour, which encloses the area of hypoxic water.

ODZs have received a lot of interest because they are nitrogen cycling hotspots. In the Baltic Sea, a substantial portion of the nitrogen (N) entering the area is converted from reactive forms to dinitrogen gas (N_2) via pelagic denitrification (Rönnér, 1983; Rönnér and Sörensson, 1985; Brettar and Rheinheimer, 1991; Hannig *et al.*, 2007; Hietanen *et al.*, 2012; Dalsgaard *et al.*, 2013, Bonaglia *et al.*, 2016). Globally, 30–50%

82 of the total nitrogen (N) loss in the oceans occurs in the ODZs (Codispoti *et al.*, 2011). Nitrification, which
83 supplies the electron acceptor for denitrification, has also been measured at high rates in the ODZs. In the
84 Baltic Sea Enoksson (1986) found potential nitrification up to $280 \text{ nmol N L}^{-1} \text{ d}^{-1}$ in a station south-west
85 from for the island of Gotland, with the highest rates occurring below the halocline. However, the rate
86 estimate may be hindered by bottle effects (i.e. senescence of cell material, which may increase the
87 availability of ammonium, (NH_4^+)) because the incubations lasted considerably longer than measurements
88 done with modern, more sensitive isotopic ratio mass spectrometers (IRMS). Bauer (2003) measured
89 potential nitrification rates of $202 \text{ nmol N L}^{-1} \text{ d}^{-1}$ in the Gotland Deep and in more recent measurements,
90 Hietanen *et al.* (2012) found potential nitrification rates of up to $160 \text{ nmol N L}^{-1} \text{ d}^{-1}$ in the Landsort Deep and
91 Berg *et al.* (2015) $130 \text{ nmol N L}^{-1} \text{ d}^{-1}$ in the Gotland Deep. Rates this high in marine water columns have
92 been detected previously only in the periodically hypoxic Bornholm Deep in the southern Baltic Sea (883.8
93 $\text{nmol N L}^{-1} \text{ d}^{-1}$; Berg *et al.*, 2015), in the Peruvian oxygen minimum zone ($144 \text{ nmol N L}^{-1} \text{ d}^{-1}$; Lam *et al.*,
94 2009), and in the Saanich Inlet ($319 \text{ nmol L}^{-1} \text{ d}^{-1}$; Grundle and Juniper, 2011).

95 Both archaeal and bacterial ammonia oxidizers can be active in ODZs. In the early 2000s, when the existence
96 of ammonia-oxidizing archaea (AOA) was unknown, the ammonia-oxidizing community in the central Baltic
97 Sea water column was suggested to be composed of β -proteobacteria (Bauer, 2003). Later on when AOA
98 were discovered, the ammonia-oxidizing community in the central Baltic Sea was suggested to consist
99 mainly of one thaumarchaeotal subcluster closely related to *Candidatus Nitrosopumilus maritimus* (Labrenz
100 *et al.*, 2010, Berg *et al.*, 2015). In the northern Baltic Sea sediments, the ammonia oxidizer communities had
101 surprisingly low diversity and were dominated by organisms with gene signatures unique to the sampling
102 area (Vetterli *et al.*, 2016). Hence, the ammonia-oxidizing communities in the Baltic Sea appear to have a
103 low diversity and harbor unique species, but the overall community composition and its controlling factors
104 are still largely unknown.

105 The diversity and community composition of ammonia oxidizers can be investigated using functional gene
106 microarrays that are designed to specifically target the ammonia-oxidizing bacteria (AOB) and AOA, using
107 sequences of their *amoA* genes, which encode ammonia monooxygenase subunit A. Since ammonia
108 oxidizers are metabolically restrained, there is very little divergence of essential genes and consequently the
109 diversity of ammonia oxidizers is relatively limited. All AOB and AOA sequences known at the time of
110 these experiments (2010–2011), both cultivated and environmental, could be targeted with this method. Each
111 microarray contains a set of archetype probes that are selected from the entire database of homologous
112 sequences, using an algorithm (Bulow *et al.*, 2008) that is similar to that used to select operational taxonomic
113 units (OTUs) (e.g. program for Defining Operational Taxonomic Units and Estimating Species Richness
114 (DOTUR); Schloss and Handelsman, 2005). Thus, each archetype represents all sequences within 85%
115 identity with the probe sequence, and the comparisons between the samples are made on the basis of relative
116 rather than absolute sequence identity because the intensity of the hybridization signal cannot be interpreted
117 quantitatively (Ward *et al.*, 2007).

118 We determined the spatial variation in the ammonia-oxidizing communities at three sites in the central Baltic
119 Sea redoxclines, using functional microarrays, to investigate how ammonia oxidizer communities are
120 composed in dynamic redoxcline where salinity and O₂ concentration in the nitrification layer change
121 frequently. We also measured the nitrification rates at four sites, created a regression model for nitrification
122 and applied it to the high resolution monitoring data that was in the IOW molecular database to estimate the
123 spatial and temporal variation of the pelagic nitrification. Thereafter, we tested whether composition of the
124 ammonia-oxidizing community correlates with the potential nitrification rates, environmental conditions
125 prevailing in the sampled areas and depths, and the differences in the hydrodynamic patterns between the
126 sampling sites. Finally, since there is interest on the pelagic denitrification and anammox due to their
127 capability to mitigate the effects of the excess N loading, we estimated how efficiently nitrification supplies
128 electron acceptors for the N₂ producing processes in this system.

129 **Materials and methods**

130 **2.1. Sample collection**

131 The samples for the nitrification rate measurements were collected from four stations during three cruises
132 2010-2011 (Table 1). Station GB1 is located at the western Gotland Basin (WGB), station LD at the
133 Landsort Deep, station GD at them Eastern Gotland Basin (EGB), and station F80 at the Fårö Deep (Figure
134 1). The microarray samples were collected in 2010 from GB1, GD, and LD (Table 1). At each of the
135 sampling stations, the salinity, temperature, and O₂ profiles were first determined, using a CTD
136 (conductivity-temperature-depth) profiler with an attached SBE43 O₂ sensor (both SeaBird Electronics Inc,
137 Bellevue, WA, USA). The oxic-anoxic interface was identified as the depth at which the signal of the O₂
138 sensor began to increase when the sensor was pulled slowly upwards after a short period on the anoxic side
139 of the redoxcline. After determining the O₂ profiles, the water samples were collected near the oxic-anoxic
140 boundary in Niskin bottles using a CTD-rosette system. Once the bottles were on deck, samples were taken
141 from two replicate bottles for potential nitrification rate measurement, microarray (only in 2010), nutrient
142 analyses (NO₃⁻, NO₂⁻, and NH₄⁺; detection limits 0.01 µmol L⁻¹, 0.01 µmol L⁻¹, and 0.3 µmol L⁻¹,
143 respectively), O₂ (Winkler titration, detection limit 0.89 µmol L⁻¹), and H₂S (detection limit 0.02 µmol L⁻¹).
144 The nutrient, O₂ and H₂S analyses followed the protocol by Grasshoff *et al.* (1983).

Table 1. The sampling stations and times, bottom and sample depths, O₂, H₂S, NO₃⁻, NO₂⁻, and NH₄⁺ concentrations, and potential nitrification rates. B/D stands for below detection limit and SE for standard error.

Station	sampling month/year	depth (m) bottom	depth (m) sample	O ₂ μmol L ⁻¹	H ₂ S μmol L ⁻¹	NO ₃ ⁻ μmol L ⁻¹	NO ₂ ⁻ μmol L ⁻¹	NH ₄ ⁺ μmol L ⁻¹	Potential nitrification rate nmol N L ⁻¹ d ⁻¹ (SE)	Microarray sample (Yes/No)
GB1	6/2010	147	57	68.3	B/D	4.70	0.03	0.5	10.1 (1.9)	Yes
	6/2010		60	49.1	B/D	4.71	0.05	0.4	11.0 (0.7)	No
	6/2010		63	20.5	B/D	4.40	B/D	0.2	31.3 (4.23)	No
	5/2011		70	12.0	B/D	4.47	0.03	0.2	30.6 (5.2)	No
	5/2011		75	0.01	B/D	0.05	B/D	2.0	1.0 (0.4)	No
LD	6/2010	453	70	4.9	B/D	2.34	0.04	0.3	79.3 (13.6)	Yes
	6/2010		73	B/D	B/D	0.45	B/D	1.6	B/D	No
	6/2010		76	3.1	4.5	0.14	B/D	3.0	5.4 (1.0)	No
	5/2011		68	9.4	B/D	5.04	0.03	B/D	22.7 (8.5)	No
	5/2011		72	1.3	B/D	0.85	1.24	1.2	81.2 (19.3)	No
GD	7/2010	242	120	0.1	B/D	4.10	0.03	B/D	75.5 (8.9)	No
	7/2010		123	1.8	B/D	B/D	B/D	0.6	3.9 (1.1)	Yes
	7/2010		126	4.5	B/D	B/D	B/D	1.3	B/D	Yes
	7/2010		130	B/D	14.2	B/D	B/D	2.9	B/D	No
	5/2011		132	B/D	9.4	1.66	0.68	0.2	43.2 (11.5)	No
	7/2011		117	5.8	B/D	5.68	0.01	B/D	14.3 (4.3)	No
	7/2011		118	7.6	B/D	5.96	0.01	B/D	B/D	No
	7/2011		119	7.1	B/D	4.41	0.06	B/D	B/D	No
F80	5/2011	191	116	0.9	B/D	1.87	0.15	0.1	B/D	No
	5/2011		120	0.9	B/D	3.47	0.06	0.2	2.2 (0.4)	No

2.2. Potential nitrification rate measurements

The potential nitrification rates were estimated by measuring the production of $^{15}\text{NO}_2^-$ and $^{15}\text{NO}_3^-$ in samples that were amended with excess $^{15}\text{NH}_4^+$. This was done by transferring a water sample from the Niskin bottle into glass bottles with a threefold overflow, and adding ^{15}N -labelled ammonium chloride ($^{15}\text{NH}_4\text{Cl}$, 99% ^{15}N , Sigma Aldrich, St. Louis, MO, USA; final concentration $\sim 5\ \mu\text{M}$ resulting in atom enrichment of 63–99-atom%) to the samples under a dinitrogen (N_2) atmosphere. The samples were then divided into 20-mL glass vials ($n = \text{nine per treatment}$) sealed gastight with butyl rubber stoppers and aluminum crimps and incubated in the dark at near *in situ* temperature ($\sim 5\ ^\circ\text{C}$). For each sample depth, three replicate samples were filtered approximately every 3–4 h through prewashed $0.2\ \mu\text{m}$ syringe filters (polyethylsulfone [PES] membrane; VWR International LLC, Radnor, PA, USA) to terminate the incubation. The maximum incubation time of the samples was approximately 9 h. The filtered samples were frozen at $-20\ ^\circ\text{C}$ for later $^{15}\text{NO}_3^-$ and $^{15}\text{NO}_2^-$ (hereafter referred to as $^{15}\text{NO}_x^-$) analysis.

The $^{15}\text{NO}_x^-$ contents of the potential nitrification rate samples were analyzed using the denitrifer method (Sigman *et al.*, 2001) with small modifications. *Pseudomonas chlororaphis* (American Type Culture Collection (ATCC) 13985) was grown in an 800-mL liquid culture (tryptic soy broth; Fluka Analytical (Sigma-Aldrich Chemie GmbH), Buchs, Switzerland), 10 mM potassium nitrate (KNO_3), 1 mM ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), and $1\ \text{mL L}^{-1}$ antifoaming agent (Dow Corning Antifoam RD emulsion; Midland, MI, USA)) on a shaker table (150 rotations per minute) for 8 d in the dark at room temperature. Thereafter, the bacterial culture was concentrated 10-fold by centrifugation and the concentrated culture was divided into 2-mL aliquots in 12-mL gastight glass vials (Exetainer; Labco Ltd, Lampeter, Ceredigion, UK). The vials were closed and purged with N_2 for 5 h. A sample amount corresponding to 8 nmol NO_x^- was injected into each vial and after overnight incubation in the dark, 0.1 mL of 10-M sodium hydroxide (NaOH) was injected into each vial to lyse the bacteria and strip the CO_2 from the headspace to the liquid. When the sample was too diluted (less than 8 nmol of NO_x^- in 5 mL), a 5-mL sample was injected into the vials to determine whether

173 minimum detectable amount (~1 nmol) of nitrous oxide (N₂O) would form. The ¹⁵N label in the N₂O
174 produced from NO_x⁻ by the denitrifying bacteria was analyzed with a gas chromatographic isotope ratio mass
175 spectrometer (GC-IRMS) system (Thermo Finnigan Delta V plus with ConFlo IV; Thermo Fisher Scientific,
176 Waltham, MA, USA) with a trace gas preconcentrator (PreCon; Thermo Fisher Scientific) in the Department
177 of Environmental Science, University of Eastern Finland, Kuopio.

178 **2.3. Microarray analyses of the amoA gene**

179 The samples for the microarray analyses were collected in 2010 from one depth at GB1 and LD and from
180 two depths at GD at the same time as the nitrification rate samples (Table 1). For each sample (n = two per
181 sampling depth), 1.5 L of water were filtered through a 0.22-μm pore-size nitrocellulose membrane filter
182 (diameter 47 mm, Durapore®; Millipore, Billerica, MA, USA) with gentle vacuum. The filters were then
183 packed in microcentrifuge tubes and frozen immediately at -70 °C for later analysis. In the laboratory, the
184 DNA from the samples was extracted, using the Qiagen Allprep kit (Qiagen, Venlo, the Netherlands) and
185 digested, using 50 ng of Hinf I restriction enzyme. Two sets of archetype probes were designed, using an
186 established algorithm (Bulow *et al.*, 2008): one for AOB (30 probes, representing 502 sequences in GenBank
187 in 2004) and a separate probe set for AOA (31 probes representing 1329 archaeal *amoA* sequences from
188 GenBank in November 2008). The resolution of the array format is about 87% +/- 3% (Taroncher-Oldenburg
189 *et al.*, 2003). Each 90-mer oligonucleotide probe consisted of a 70-mer archetype sequence combined with a
190 20-mer reference oligo as an internal standard. Targets for microarray hybridization were prepared,
191 hybridized in duplicate on the microarray slide, and washed as described in Ward and Bouskill (2011). The
192 washed slides were scanned, using a laser scanner 4200 (Agilent Technologies, Palo Alto, CA, USA) and
193 analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA, USA). All of the original array files are
194 available at GEO (Gene Expression Omnibus; <http://www.ncbi.nlm.nih.gov/geo/>) at NCBI (National Center
195 for Biotechnology Information) under GEO Accession No. GSE50164.

Quantification of the hybridization signals was performed according to Ward and Bouskill (2011). The initial data are in the form of a fluorescence ratio (FR), the cyanine 3/cyanine 5 (Cy3/Cy5) ratio, for every feature. The FR values were converted to a relative fluorescence ratio (RFR), which is the fraction of total fluorescence (sum of all the FR values for each probe set) for each probe. Hence, the final results are relative hybridization strengths, not absolute abundances.

2.4. Calculations and statistical analyses

The potential nitrification rate was calculated by plotting the change in average NO_x^- concentrations over the incubation time (Jäntti *et al.*, 2013). The slope of this equation represents the nitrification rate and the rate was determined as significant when in linear regression analysis $P < 0.05$. The change in the NO_x^- concentration for each time point was calculated according to equation 1:

$$(1) \text{NO}_x^- = [\text{NO}_x^-] \times (\Delta\text{atom}\%/100) / R_{\text{NH}_4^+}$$

where $\Delta\text{atom}\%$ is the difference between the atom% of NO_x^- at the time point and in the beginning of the incubation and $R_{\text{NH}_4^+}$ is the ^{15}N enrichment in the NH_4^+ pool after the addition of $^{15}\text{NH}_4^+$. To extrapolate the potential nitrification rates for the entire central Baltic Sea, the rates and the environmental variables from this study and Hietanen *et al.*, (2012) were combined and a stepwise multiple regression analysis was performed with Sigmaplot statistic program (Systat, San Jose, CA, USA). The rates measured in zero O_2 concentration were excluded from the regression analysis due to high variability of rates that was probably caused by some of the samples having H_2S and O_2 below the detection limit of the Winkler method. To calculate the nitrification rates in the redoxcline, the regression model was applied to three independent data sets collected in 2009, 2010, and 2011 by Frey *et al.*, (2014), where the O_2 and dissolved inorganic nitrogen (DIN) concentrations were analyzed with a high vertical resolution in the central Baltic Sea. To extrapolate the rates for the entire central Baltic Sea, the thickness and the depth of the active nitrification layer was

218 calculated from the IOW molecular biological data base, which contains vertically highly resolved DIN and
219 O₂ data collected from the central Baltic Sea during five IOW monitoring cruises 2008-2012 (FS Maria S.
220 Merian 08, June and August 2008; FS Alkor 332, February-March 2009; FS Maria S, Merian 12, August-
221 October 2009; FS Meteor 86, November-December 2011; FS Meteor 87, May-August 2012). The thickness
222 and the depth of the nitrification layer for each cruise was computed with gradient method by restricting the
223 NO₃⁻ concentration between 0-6.0 μM, O₂ concentration between 0-25.0 μmol L⁻¹ and NH₄⁺ concentration
224 between 0-1.0 μM. These concentration limits were chosen because in the Baltic Sea H₂S typically
225 accumulates almost immediately beneath the water layer where O₂ concentrations is below detection limit,
226 and inspection of the profiles showed that the NO₃⁻ peak, which is considered to be at the top of the active
227 nitrification layer, typically falls between these limits. Also, the highest ammonia oxidizer gene activity has
228 been shown to fall in between these limits (Labrenz *et al.* 2010). A careful inspection of the position of the
229 anoxic layer indicated that the 70 m depth contour is representative for the area of redoxcline. The area
230 surrounded by the 70 m depth contour was computed using the Matlab (Mathworks Natick, MA, USA)
231 function trapz(x,y), which provides a trapezoidal numerical integration of data with non-uniform spacing,

232 The diversity of the microbial communities was estimated by calculating the Shannon evenness index. The
233 Bray-Curtis dissimilarity index was calculated, using R (R Core Team 2012). Redundancy analysis (RDA)
234 was performed in R, using the RFR of each archetype (after square-root transformation) as the response
235 variables, and dissolved O₂, NO₃⁻, and NH₄⁺ concentrations and potential nitrification rates (at the microarray
236 sample depth) as explanatory variables.

237 **3. Results**

238 **3.1. The environmental conditions during nitrification rate measurements**

239 The oxic-anoxic interface was between 70–126 m and mixing of oxic and euxinic water masses was evident
240 on some occasions at GD and LD where both H₂S and O₂ existed in the same water layers (Table 1). The O₂
241 concentration in the sampling depths was 0–70 μM, NH₄⁺ concentration 0–3 μM and NO₃⁻ concentration 0–6
242 μM (Table 1). Substantial NO₂⁻ accumulation was observed only on few sampling occasions (Table 1).

243 3.2. Nitrification rates

244 The highest potential nitrification rates (76–81 nmol N L⁻¹ d⁻¹) were measured at stations GD and LD at
245 depths where O₂ was still present, but at low concentrations (Table 1). The NO_x⁻ concentration did not
246 increase linearly over the incubation period in 73 m at LD; in 126 m, 130 m, 118m, and 119 m at GD; and in
247 116 m at F80 (Table 1). Data from these measurements were discarded from further analyses. The non-
248 linearity was most likely caused by the low nitrification rates approaching the detection limit of the method.

249 The highest significant (p = 0.0008) R-value (0.6917) in the regression analysis was obtained for the
250 equation where logarithmic potential nitrification rate had a quadratic relationship with the logarithmic O₂
251 concentration (Equation 2, Figure 2).

$$252 \quad (2) \log(\text{nitrification rate}) = -0.8447(\log(O_2))^2 + 1.711 \log(O_2) + 0.7934$$

253 There was also a significant linear negative correlation between the nitrification rate and NH₄⁺
254 concentrations but the R-value (0.4262) was lower than for equation 2. No significant correlation was found
255 when both O₂ and NH₄⁺ were included in to the analysis.

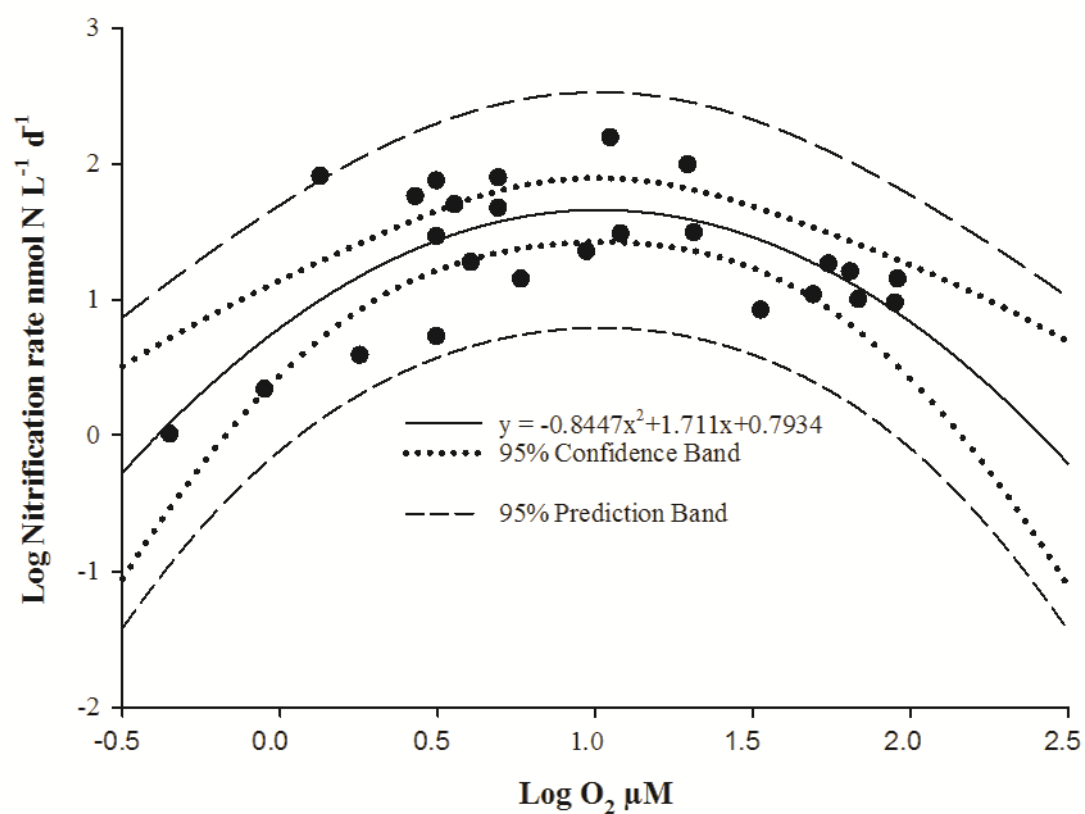
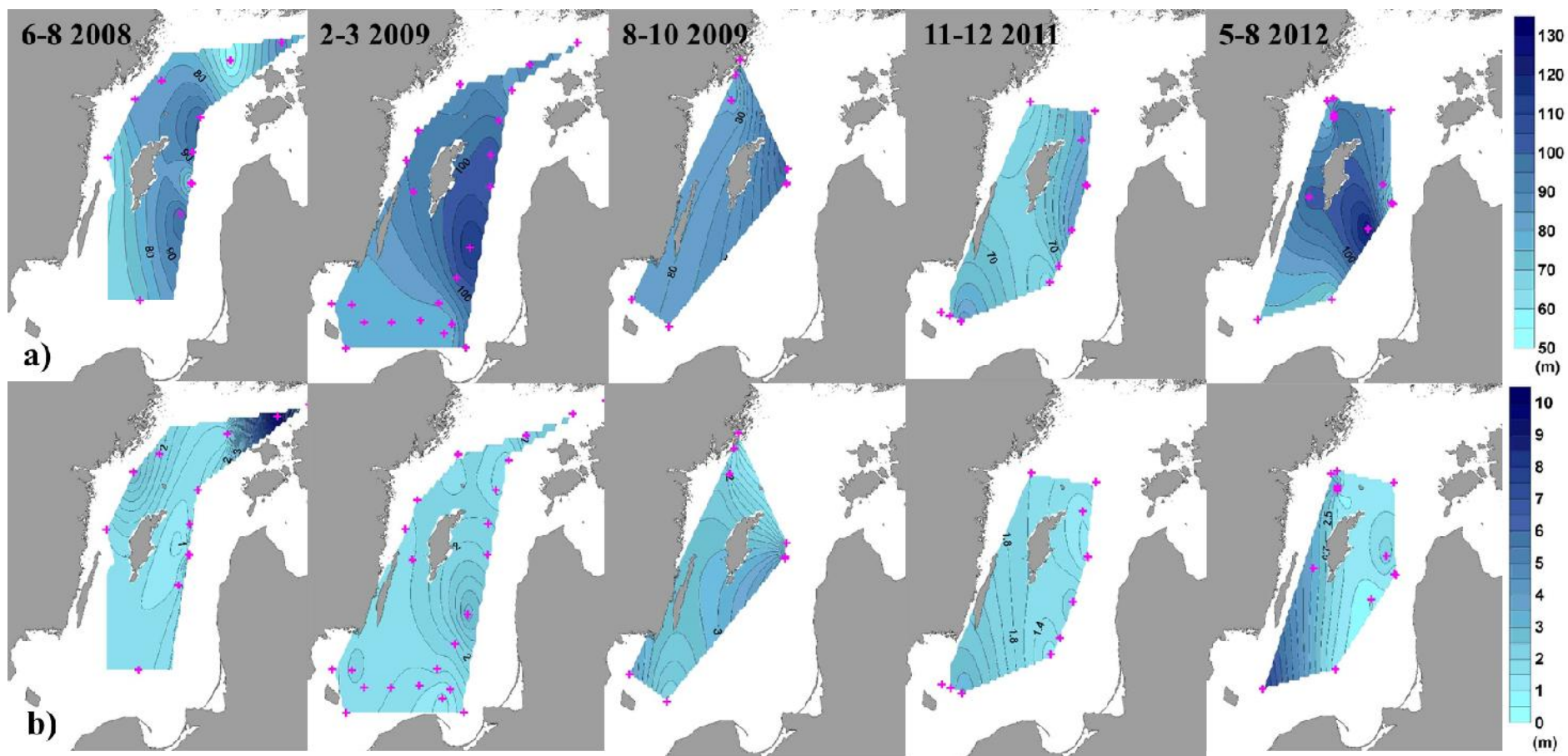


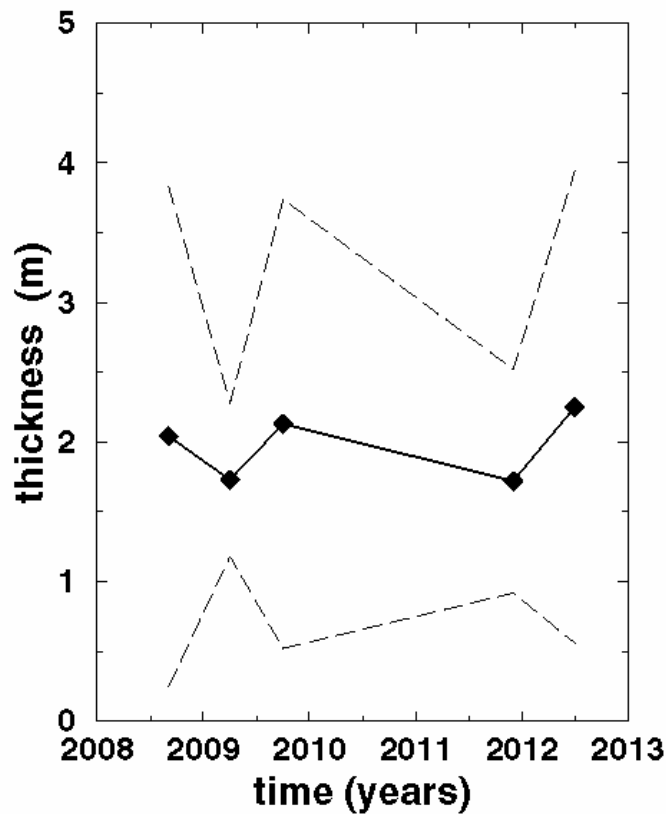
Figure 2. The regression model for nitrification rates in the Central Baltic Sea water column.



256

257 **Figure 3.** The depth of the center of the nitrification layer (a) and thickness (b) of the nitrification layer 2008-2012. Data was compiled from the IOW
 258 monitoring database.

259 The modeled nitrification rates in redoxclines were $39.9 \pm 3.6 \text{ nmol N L}^{-1} \text{ d}^{-1}$ (2009), $38.5 \pm 6.3 \text{ nmol N L}^{-1} \text{ d}^{-1}$
 260 1 (2010), and $35.9 \pm 11.7 \text{ nmol N L}^{-1} \text{ d}^{-1}$ (2011). The average depth of the modelled nitrification layer was 83
 261 $\pm 18 \text{ m}$ at GD, $77 \pm 11 \text{ m}$ at LD and 75.4 for F80 and the thickness of the nitrification layer varied between
 262 $0.86\text{--}3.11 \text{ m}$ in the sampling stations (Figure 3, Table 2). There are no data available to compute the depth of
 263 the nitrification layer at GB1 and only one time point for F80 (Table 2). The area suitable for nitrification to
 264 proceed in the water column was approximately $77,540 \pm 1000 \text{ km}^2$ and multiplying this area with the
 265 average thickness of the water layer suitable for nitrification ($2.04 \pm 1.40 \text{ m}$ (Figure 4)), and the average
 266 nitrification rate from the equation, results an approximate annual amount of nitrification of $30.07 \pm 21.64 \text{ kt}$
 267 of N.



268 **Figure 4.** The average thickness and the standard deviation of nitrification layer in the central Baltic Sea
 269 2008-2012.

Table 2. The depth/thickness of the nitrification layer (m) in 2008-2012 at GD, LD and F80. No data for GB1 is available in the IOW monitoring database.

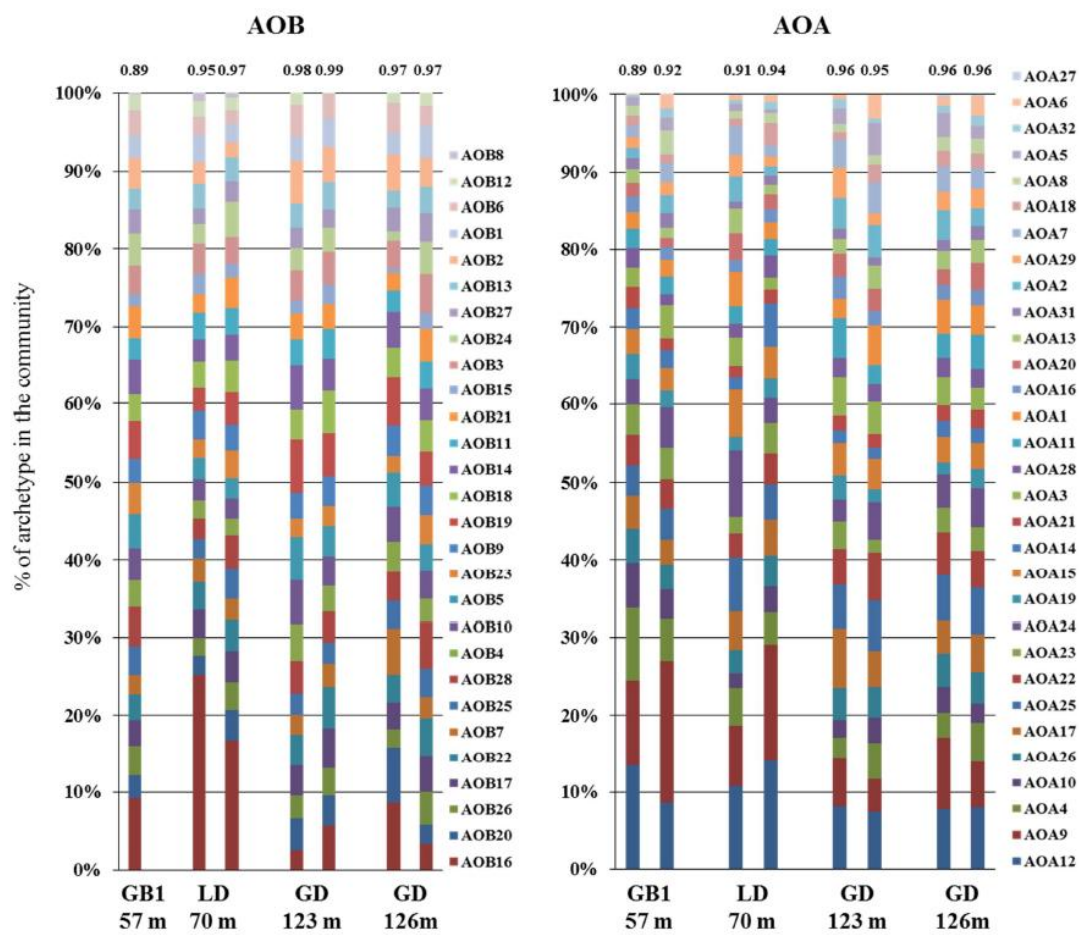
	F80	LD	GD
6–8, 2008	N/A	85.40/2.88	59.41/1.19
2–3, 2009	N/A	N/A	99.51/1.95
8–10, 2009	N/A	77.58/2.19	92.54/2.87
11–12, 2011	75.36/1.01	62.79/1.60	80.36/0.86
5–8, 2012	N/A	81.69/3.11	83.17/2.46
AVERAGE	75.36/1.01	76.87/2.46	83.00/1.87
STD	-	11.48/0.69	17.58/0.84

3.3. Ammonia-oxidizing organism community composition

The Bray-Curtis dissimilarity index (0.05–0.19) for each replicate pairwise comparison indicated substantial variability between the replicates. However, the samples in general did cluster by pairs of replicates. For station GB1, only one sample was included for the AOB analysis, because the replicate sample did not hybridize well and the results were discarded. Overall, the archetypes for both AOA and AOB were quite evenly distributed (Figure 5) and the Shannon evenness index varied between 0.89 and 0.99 (Figure 5). The AOB and AOA communities at GB1 were the least even (Shannon evenness index 0.89), indicating that there were some archetypes that were relatively more important than others at this station.

For AOB, the highest signal archetype at GB1 as well as at LD was AOB16. The other important archetypes were AOB20 and AOB26 (Figure 5). For the AOA, there were three somewhat disproportionately important archetypes at all stations: AOA9, AOA12, and AOA4 (Figure 5). The RDA indicates that the AOB and AOA communities at GD clustered furthest away from the communities at GB1, whereas the communities at LD were located between GB1 and GD (Figure 6). The samples from the GD 123 m and 126 m were relatively similar indicating that although the potential nitrification rates declined, the ammonia oxidizer community did not change (Table 1, Figure 6). There was surprisingly wide variation between the replicate samples at LD; however, no errors were found in the analytical procedure, so both replicates were included in the analysis.

288 The AOB16 archetype was highly correlated with the potential nitrification rates and, therefore, with the
289 samples from LD, where the rates were highest (Figure 6). If AOB are important at all in this system, this
290 archetype is probably the most important, based on its high relative abundance and correlation with the
291 potential nitrification rate. AOB7, AOB17, AOB20, AOB22, and AOB27 all showed their highest RFR
292 signals at GB1, the sample that had the highest O₂ concentration (Figure 6). Hence, these archetypes were
293 probably associated with higher O₂ concentrations. None of the other AOB archetypes showed any striking
294 patterns. AOA4 and AOA12 showed the highest signals at GB1, while AOA9 showed high signals at both
295 the GB1 and LD stations (Figure 5). AOA14 was correlated with potential nitrification rate and showed its
296 highest signal in the first replicate at LD, but was moderate in the second. Hence, there was poor replication
297 between the samples. AOA3 and AOA5 showed consistently high signals at both depths sampled at GD and
298 were correlated with NH₄⁺, which was highest at 126 m at GD (Figure 6).



299 **Figure 5.** Distribution of archetypes based on relative fluorescence ratio (RFR) signals. The Shannon
300 evenness index is presented on top of the bars.

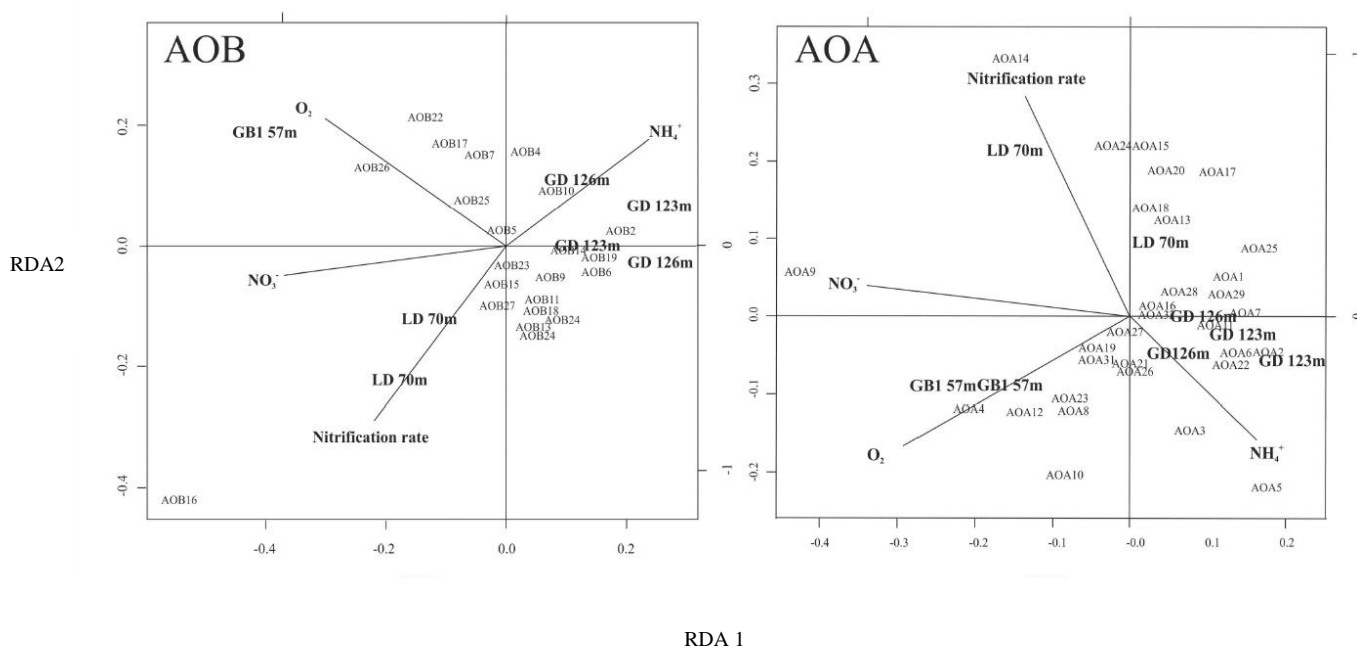


Figure 6. Redundancy analysis (RDA) maps of the AOB and AOA, sampling stations, and environmental parameters.

4. Discussion

4.1. Nitrification rates in the redoxclines

The potential nitrification rates measured in this study suggest that the maximal rates in the central Baltic Sea occur right above the oxic-anoxic interface and the rates decrease to zero quickly above and below that (Table 1). This was particularly demonstrated in the samples that were taken at LD in 2010. At 70 m the potential was at its highest but the rates quickly decreased below detection limit by 73 m, the depth where O_2 was not present anymore. However, at 76 m there was again O_2 and nitrification potential commenced above detection limit. Hence, it appears that nitrification does not only proceed in a uniform layer but also in lenses that contain O_2 below the oxic anoxic interface. The presence of O_2 at GD in 2010 fluctuated similar to LD, but nitrification did not initiate at 126 m although O_2 concentration increased slightly from 123 m. Hence,

314 the presence of nitrification at these lenses may be regulated also other factors than O₂, such as proximity of
315 H₂S, which is known to inhibit nitrification in the pelagic Baltic Sea (Berg *et al.* 2015).

316 The calculated water layer where conditions are favorable for nitrification is surprisingly narrow and there
317 was very little variation between the areas and years (Figure 3, Figure 4). We always tried to target the most
318 active nitrification layer based on the inspection of O₂ profile, yet the rates were often below detection limit
319 or very low, indicating that we may have missed the most active layer (Table 1). When Labrenz *et al.* (2010)
320 measured the ammonia oxidizer gene expression they found, similar to us, the highest activity in a two
321 meters thick water layer at the oxic anoxic boundary. The reason for the thin nitrification layer in the Baltic
322 Sea is probably the lack of extended suboxic zone where conditions are favorable for pelagic nitrification
323 (Lam *et al.* 2007, Lam *et al.* 2009, Kalvelage *et al.* 2011, Bristow *et al.* 2016) and which is a prominent
324 feature of many other ODZs such as the Black Sea (Yakushev *et al.* 2008), the Eastern Tropical Pacific
325 OMZ's (Paulmier *et al.* 2006) and the Saanich Inlet (Zaikova *et al.* 2009). The narrow suboxic layer is also
326 consistent with very low anammox and N₂O production rates in the Baltic Sea. Anammox is inhibited by H₂S
327 and it occurs at significant rates in the Baltic Sea only after inflows when H₂S has not reached the suboxic
328 layer (Hannig *et al.*, 2007, Bonaglia *et al.*, 2016). Similarly, substantial N₂O formation, which results from
329 nitrification in suboxic conditions, has been found only after inflows when sulfidic waters have not reached
330 the oxic anoxic interface (Myllykangas *et al.*, 2017). Observations in the Bornholm Basin in the southern
331 Baltic Sea (van der Lee and Umlauf, 2011) indicate that higher modes of the near-inertial wave spectrum are
332 generated at the slope of the basin and they create persistent narrow shear band. These perturbations
333 propagate in to the EGB from the edge of the basin into its interior at the redoxcline (Holtermann *et al.*,
334 2017). The narrow bands of high shear are directly associated with narrow bands of dissipation, the major
335 source of turbulent mixing (Lappe and Umlauf, 2016) that prevents the formation of the thick suboxic layer.
336 This also explains the formation of O₂ containing lenses, which harbors nitrification below the oxic anoxic
337 interface.

338 The depth of the nitrification layer was between 59–100 m in at GD and 63–85 m at LD. Hence, the depth of
 339 the nitrification layer varied more at GD (Figure 3, Table 2). Although there were no MBIs during the
 340 analysis period, the position of the nitrification layer appears to fluctuate substantially particularly in the
 341 EGB (Figure 3). The dynamic nature of the nitrification layer in this area may be explained by minor inflows
 342 that occurred during the analysis period (Naumann *et al.*, 2016). The minor inflows are not strong enough to
 343 replace old anoxic water in the bottom of the basins. Instead, they mix with the intermediate water layers and
 344 cause entrainment of the water column. The minor inflows propagate first into the EGB before traveling into
 345 the WGB. As the inflowing water travels through the EGB, its salinity decreases when the water masses mix
 346 with less saline water. Consequently, the inflow weakens and may not necessarily reach the WGB at all.
 347 Therefore, WGB has less frequent and weaker lateral intrusions and a more stable redoxcline (Matthäus *et*
 348 *al.*, 2008), which also appears to cause the depth of the nitrification layer to remain more stable (Figure 3,
 349 Table 2).

350 **4.2. Nitrification as a regulatory factor for nitrogen removal in the Baltic Proper** 351 **redoxclines**

352 Denitrification is an important sink for NO_x^- in the central Baltic Sea and it has been estimated to remove
 353 132–547 kton N yr^{-1} (Dalsgaard *et al.*, 2013). We estimated that nitrification produces approximately 30 kton
 354 of N yr^{-1} , which is less than a quarter of the lowest denitrification estimate. In order for nitrification to match
 355 the denitrification rates estimated by Dalsgaard *et al.*, (2013) the average nitrification rate at the entire
 356 central Baltic Sea would have to be approximately 170 $\text{nmol N L}^{-1} \text{ d}^{-1}$ which still is within the 95%
 357 prediction interval of the regression model (Figure 2). Such high rates have also been measured in the area
 358 (Hietanen *et al.*, 2012), but based on our measurements and model, they are unlikely to be maintained
 359 throughout the year in the entire area. Hence, although there is a strong coupling between nitrification and
 360 denitrification in the central Baltic Sea (Frey *et al.*, 2014), there are probably additional sources of NO_3^- for
 361 denitrification. Such sources could be nitrification occurring in lenses formed by mixing and lateral transport

362 of NO₃⁻ by advection. However, their importance as NO₃⁻ source for denitrification needs further
363 investigations.

364 **4.3. Community composition of ammonia-oxidizing organisms in the central Baltic** 365 **Sea**

366 The high signals for AOB16 and AOB20 were consistent with the origin of these archetype sequences and
367 the characteristics of the Baltic environment. The archetype sequence of AOB16 is from Kysings Fjord, a
368 small coastal lagoon in Denmark (Nicolaisen and Ramsing, 2002). Kysings Fjord is characterized by high N
369 loads, salinity of 14, and virtually no tidal action (Nielsen *et al.*, 1995). This archetype was also associated
370 with high potential nitrification rates, so the most active AOB in the Baltic Sea probably cluster closely with
371 this archetype. The sequence of AOB20 is based on *N. cryotolerans*, which was originally isolated from cold
372 waters in Alaska and is capable of growth even at temperatures of -5 °C (Jones *et al.*, 1988). Although the
373 temperature in the sampling depth was cool (~5 °C), the appearance of this archetype is not necessarily tied
374 to temperature, since the archetype is universally distributed. For example, this sequence was retrieved in a
375 wastewater treatment plant in Japan (Limpiyakorn *et al.*, 2005). The rarer archetype among the highest
376 signals was AOB26 (Figure 5). This archetype sequence was derived from Gulf of Finland sediments located
377 in the northern Baltic Sea and it has been detected elsewhere (e.g. Chesapeake Bay, Bouskill *et al.*, 2011),
378 but not as a major component of the assemblage. Therefore, the high relative abundance of AOB26 seems to
379 be specific for the Baltic Sea and is in line with the results of Vetterli *et al.*, (2016) indicating that the Baltic
380 Sea harbors unique ammonia oxidizer sequences.

381 The AOA microarray results showed no striking patterns specific for the Baltic Sea. Similar high relative
382 abundances for AOA9, AOA12, and AOA4 have been shown in other studies in which AOA microarrays
383 were applied for marine samples (Bouskill *et al.*, 2012, Newell *et al.*, 2013). The sequence for AOA9 was
384 derived from deep low-O₂ water samples from the Gulf of California and has also been detected in deep

385 water from Monterey Bay and off Hawaii at station ALOHA. While the Baltic Sea redoxcline, too, shows
386 low O₂ conditions, the Baltic Sea is relatively shallow, and the low O₂, rather than depth, appears to regulate
387 the presence of this archetype. The sequence for archetype AOA12 was compiled from sequences derived
388 primarily from representatives of Tobarí sediments, a hypernutrified estuary in Mexico, and from clones that
389 are derived from soil. The sequence for AOA4 was derived from *N. gargensis* and sequences representing
390 soil and sediment. Although AOA12 and AOA4 were associated with soil and sediment, these archetypes are
391 also commonly found in marine water columns (Bouskill *et al.*, 2012; Newell *et al.*, 2013). Interestingly, the
392 high relative abundance of these three archetypes appears not to be dependent on salinity, because they have
393 been found under completely marine conditions (Newell *et al.*, 2013), as well as the brackish water
394 conditions that were present in this study.

395 AOA1 was not among the archetypes that showed high signal strength (Figure 5), although its probe
396 sequence is derived from *N. maritimus* and should be closely related to AOA cluster GD2, detected at high
397 abundance in the Baltic by Labrenz *et al.* (2010) and Berg *et al.* (2015). This suggests that the GD2 cluster
398 *amoA* sequences did not hybridize with the AOA1 probe because the sequence fragments published by
399 Labrenz *et al.* (2010) only partially overlap with the AOA1 probe sequence and that GD2 is not closely
400 related to *N. maritimus*. The GD2 *amoA* sequence appears to be only about 90% identical to the AOA1 probe
401 sequence and this degree of similarity between target and probe would produce low signals even if the
402 mismatched target were abundant. Hence, it appears that the dominant thaumarchaeotal subcluster in the
403 Baltic Sea has evolved a unique lineage that is adapted to the varying salinity, and O₂ and H₂S
404 concentrations. If the GD2 sequence had been available at the time of the array design, it probably would
405 have constituted a distinct new archetype probe, the inclusion of which in the microarray could have shifted
406 the diversity of the AOA archetypes to a less even distribution. Nevertheless, the comparisons are made on
407 the basis of relative contribution to the assemblages in different samples and their relationship to
408 environmental variables remain valid.

4.4. Effect of water column hydrodynamics on nitrifying communities

In microarray analyses, the number of types detected is limited by the number of probes; hence the diversity index (number of species) is not a proper measure of diversity. Instead, the evenness index should be used. In this study, the overall species evenness was higher than anywhere else where ammonia oxidizer assemblages have been analyzed using a similar method (Ward *et al.*, 2007; Bouskill *et al.*, 2011, 2012; Newell *et al.*, 2013). The high degree of evenness in the AOA and AOB communities may be explained by the unique physical features of the Baltic Sea that cause disturbances to the water layers where ammonia oxidizers are present. The intermittently occurring MBIs and the frequent turbulent mixing in the redoxcline causes variation in salinity, which has been suggested to be one of the main drivers for the diversity of ammonia oxidizers (Bernhard *et al.* 2005). Mixing also alters the geochemistry, which is a major driver for the OTU distribution (Bouskil *et al.* 2012). Mixing of the water column is more prominent in the EGB than in the WGB (Matthäus *et al.*, 2008, Dellwig *et al.*, 2012, Jakobs *et al.*, 2013) (Figure 3) and the more stable redoxcline at GB1 may allow the most adapted species to dominate the ammonia oxidizer community, which is consistent with the less even distribution of archetypes at that station.

Physical processes, such as turbulence and advection, control salinity and the distribution of geochemical components. Since salinity and geochemical components are highly correlated with the compositions and activity levels of microbial communities, they also govern the biological cycling of geochemical components. This study is a modest attempt to demonstrate this and in the changing climate, even more thorough combination of biological and hydrodynamic data is required in order to understand the future projections of the biogeochemical cycles.

431 **Conclusions**

432 The nitrification rates in the central Baltic Sea are at their highest in the upper redoxcline and quickly
433 decrease below detection limit a few meters below and above the most active layer. This is caused by the
434 lack of an extensive suboxic zone, which is a prominent feature of many other ODZs. There is very little
435 temporal variation in the average nitrification rates and the average thickness of the nitrification layer. The
436 limited size of the persistent nitrification layer might be directly associated to the turbulent mixing. Higher
437 modes of near-inertial gravity waves create narrow bands of high shear and dissipation and such a permanent
438 physical forcing seems to be sufficient to form the thin and persistent nitrification layer. However, the depth
439 of the water layer where conditions are suitable for nitrification had more variability in the EGB than in the
440 WGB. The thin nitrification layer highlights the uniqueness of the hydrodynamics in the Baltic Sea and its
441 effects on the nitrification rates – the volumetric rates are some of the highest measured pelagic redoxclines,
442 yet the areal rates are low because the conditions favourable for nitrification are found only in a narrow
443 water layer. The turbulent conditions in the redoxcline also seem govern the ammonia-oxidizing community
444 composition because the community is more evenly distributed than observed elsewhere where functional
445 micro-arrays have been applied. The ammonia-oxidizing community in the EGB is more even than in the
446 WGB and the reason for the more even community composition is most likely the more dynamic redoxcline
447 where environmental conditions change constantly, allowing no predominance of single ammonia-oxidizing
448 archetype.

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